ARTICLE

The Aryl Hydrocarbon Receptor (AhR) and Its Nuclear Translocator (Arnt) Are Dispensable for Normal Mammary Gland Development but Are Required for Fertility

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Summary: The aryl hydrocarbon receptor (AhR) and its nuclear translocator (Arnt) are transcription factors that play a role in the detection of and adaptation to environmental signals. AhR-null mice are viable but show impaired lactation. Deletion of the *Arnt* gene from the mouse genome results in embryonic lethality. To determine the role of Arnt in mammary development and function, we inactivated the *Arnt* gene in mammary epithelium using Cre-*loxP* recombination. Inactivation of the *Arnt* gene during pregnancy did not disrupt alveolar development or the ability of dams to nurse their litters. In contrast, dams in which the *Arnt* gene had been inactivated during puberty and in ovaries were subfertile, exhibited retarded mammary development, and impaired mammary function. To distinguish defects autonomous to mammary epithelium from indirect effects controlled by ovarian hormones, we transplanted Arnt-null and AhR-null mammary epithelium into wild-type mice and evaluated development after one pregnancy. Normal mammary structures were observed in the absence of Arnt and AhR, demonstrating that neither transcription factor is necessary for mammary development. genesis 32:231–239, 2002.   © 2002 Wiley-Liss, Inc.

Key words: mammary glands, ovary, mouse, cre, loxP, aryl hydrocarbone receptor

INTRODUCTION

The aromatic hydrocarbon receptor (AhR) and its obligate heterodimerization partner, aromatic hydrocarbon receptor nuclear translocator (Arnt), are members of the basic-helix-loop-helix, Per/Arnt/Sim (bHLH-PAS) family of transcription factors that play a role in the cell's ability to respond to environmental cues, including developmental signals (Gu *et al.,* 2000). Environmental toxicants, including 2-, 3-, 7-, 8-tetrachlorodibenzo-p-dioxin (TCDD) are known to activate the AhR-Arnt pathway. Adult female animals exposed to TCDD exhibit a general decrease in reproductive health, including decreased fertility and litter size, alterations in estrous/menstrual cycles, and an increased abortion rate (Li *et al.,* 1995; Peterson *et al.,* 1993). Although no endogenous ligand has been identified, a case can be made that the AhR-Arnt pathway supports ovarian function and cell proliferation required for functional mammary gland development. AhR-null mice are viable (Fernandez-Salguero *et al.,* 1995; Schmidt *et al.,* 1996), and analyses of mammary development revealed a reduction in terminal end buds (Hushka *et al.,* 1998). In the ovary, AhR influences the rate of primordial follicle formation and the number of antral follicles (Benedict *et al.,* 2000). Deletion of the *Arnt* gene results in embryonic lethality (E10.5) due to a defect in angiogenesis and placental function (Kozak *et al.,* 1997; Maltepe *et al.,* 1997).

Mammary development during puberty and pregnancy is dependent on ovarian steroid hormones, including estrogen (Korach *et al.,* 1996) and progesterone (Lydon *et al.,* 1995). Whereas estrogen is required for ductal outgrowth and branching during puberty, progesterone is required for alveolar development. Since the AhR-Arnt and estrogen receptor pathways are closely linked (Safe *et al.,* 2000), we hypothesized that Arnt and AhR could play a role in the development and differentiation of mammary tissue. To explore the function of Arnt in the development of mammary tissue, we used

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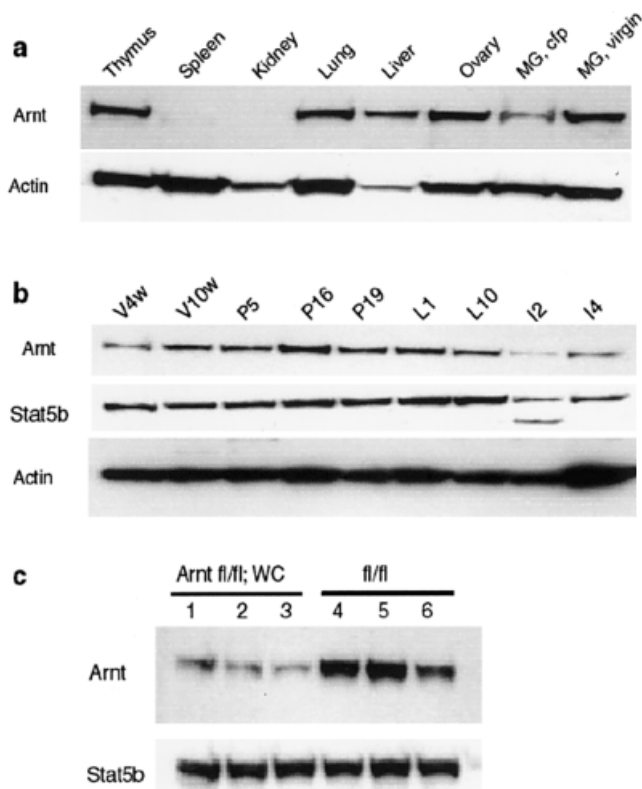 E-mail: hennighausen@nih.gov

FIG. 1. Steady state levels of Arnt in mouse tissue **(a),** during mammary gland development **(b),** and upon inactivation of the *Arnt* gene **(c).** (a) Proteins from thymus, spleen, kidney, lung, liver, ovary, cleared mammary fat pad (cfp), and mammary tissue from a virgin (MG) were analysed with anti-Arnt and anti-actin antibodies. (b) Proteins from mammary tissue were analysed from 4-week-old (V4w) and 10-week-old (V10w) virgins, days 5 (P5), 16 (P16), and 19 (P19) of pregnancy, days 1 (L1) and 10 (L10) of lactation, and days 2 (I2) and 4 (I4) of involution. Arnt, Stat5b, and actin protein levels were analysed using the respective antibodies. (c) Arnt levels were analysed in mammary tissue from Arnt fl/fl; WC (lanes 1–3) and Arnt fl/fl (lanes 4–6) mice at day 5 of lactation. Arnt and Stat5b protein levels were analysed by Western blot.

the Cre-*loxP* system to inactivate the *Arnt* gene in mammary epithelium and ovary. This was accomplished with Cre transgenes under control of the whey acidic protein (WAP) gene promoter and the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (Wagner *et al.,* 1997, 2001).

RESULTS

Expression Profile of Arnt in Mammary Tissue

Arnt RNA is expressed in several tissues (Hirose *et al.,* 1996), but steady state protein levels have not been investigated. We now measured Arnt levels in thymus, spleen, kidney, lung, liver, ovary, and mammary tissue using Western blot analyses (Fig. 1a). Arnt was detected in all tissues with the exception of spleen and kidney, and it was present in both mammary epithelium and in the stroma. We further established the presence of Arnt

throughout mammary development. Arnt was detected during puberty, pregnancy, lactation, and involution (Fig. 1b). The levels were lowest in 4-week-old virgin mice and during involution.

Deletion of the *Arnt* Gene from Mammary Epithelium

Mice in which exon 6 of the *Arnt* gene was flanked by *loxP* sites (Tomita *et al.,* 2000) were mated with transgenic mice that carry the Cre gene under control of either the MMTV-LTR or the WAP gene promoter (Wagner *et al.,* 1997). While the MMTV-Cre mice express Cre recombinase in ductal epithelium of virgin mice and in alveolar epithelium throughout pregnancy (Wagner *et al.,* 2001), the WAP-Cre gene is active in differentiating alveolar cells during late pregnancy and lactation (Walton *et al.,* 2001). We utilized these characteristics in expression kinetics to inactivate the *Arnt* gene in specific compartments and during different developmental stages. Mice were generated that carried two floxed Arnt alleles (fl/fl) and the MMTV-Cre or WAP-Cre transgene. These mice will subsequently be referred to as Arnt fl/fl; MC and Arnt fl/fl; WC. To evaluate the loss of Arnt, we performed Western blot analyses of mammary tissue from Arnt fl/fl; WC mice at day 10 of lactation (Fig. 1c). Arnt levels were reduced by approximately 80% (Fig. 1c). This finding is in agreement with studies employing ROSA 26 mice that suggest a recombination in approximately 80% of the epithelium (Walton *et al.,* 2001). The residual Arnt signal can be attributed to stromal expression as well as the epithelium that had failed to undergo Cre-mediated recombination of the *Arnt* gene.

Arnt Is Not Required for Mammary Alveolar Development, Differentiation, and Function

To investigate the role of Arnt in the differentiation and function of mammary alveolar cells during pregnancy and lactation, its gene was inactivated with the WAP-Cre transgene, which is active in differentiating mammary epithelial cells during pregnancy and during the lactation. Normal mammary development was observed in the absence of Arnt (Fig. 2) and dams could nurse their litters (Fig. 3). The alveolar compartment filled the fat pad and milk was produced. These mice were able to raise their litters even after several pregnancies (data not shown).

Arnt Is Not Required for the Development of Mammary Ducts

To investigate whether Arnt contributes to the development of ductal epithelium during puberty, the gene was inactivated using the MMTV-Cre transgene. In the absence of Arnt, mammary ducts exhibited normal elongation and branching during puberty (Fig. 4). However, the litter sizes of Arnt fl/fl; MC dams were small (4 ± 2 pups) and 60% of the dams could not support their young. When the pups survived, they were smaller than pups nursed by Arnt fl/fl dams. Histologic evaluation of
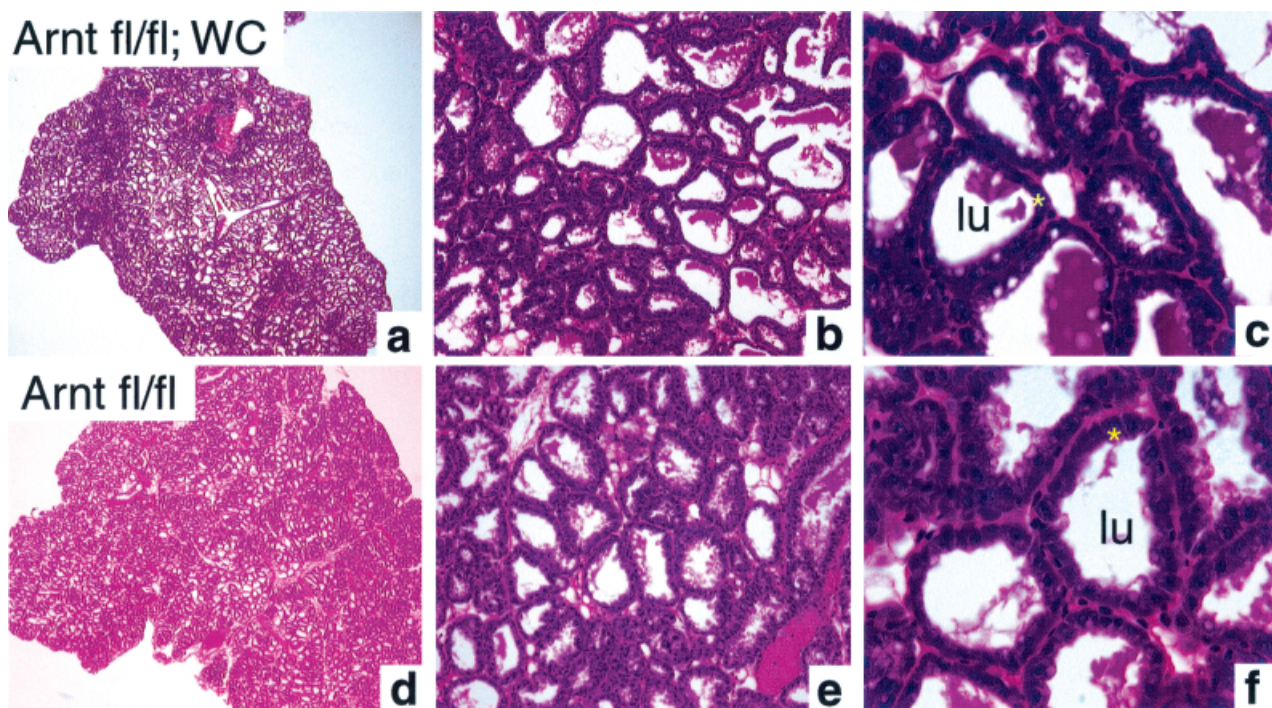
FIG. 2. Histological analyses of mammary tissue from Arnt fl/fl; WC and Arnt fl/fl mice. Mammary tissues from Arnt fl/fl; WC **(a–c)** and Arnt fl/fl **(d–f)** mice were harvested at day 5 of lactation. Magnification 25× (a,d); 200× (b,e); 630× (c,f). lu: lumen; *, epithelial cell.

Arnt fl/fl; MC mammary epithelium after parturition revealed incomplete alveolar development (Fig. 4).

MMTV-Cre mice express Cre recombinase not only in mammary epithelium but also in other cell types, including the ovary (Wagner *et al.,* 1997, 2001). Since functional ovaries are required for normal mammary gland development during pregnancy, we investigated whether the incomplete mammary development, the impairment of lactation, and the small litter sizes observed in the Arnt fl/fl; MC dams were possibly a secondary effect due to subfunctional ovaries. For this purpose, we transplanted mammary epithelium from Arnt fl/fl; MC virgin mice into athymic nude mice and evaluated mammary development at parturition. At day 1 of lactation, Arnt fl/fl; MC mammary epithelium had developed normally, and the fat pad was filled with secretory alveoli (Fig. 5), suggesting that the lesions observed in Arnt fl/fl; MC were of secondary nature.

A Role for Arnt in the Ovary

Western blot analysis established that Arnt is present in ovaries (Fig. 1a). The line of MMTV-Cre transgenic mice used in our study express Cre recombinase in oocytes, granulosa cells, and the corpus luteum (Riedlinger *et al.,* 2002; Wagner *et al.,* 2001). We performed morphometric analyses on ovaries from 3-month-old Arnt fl/fl; MC (n=2) and Arnt fl/fl females (n=4). Ovaries from Arnt fl/fl; MC mice contained less primordial follicles (6,640 ± 2,942) than those from Arnt fl/fl mice (13,000 ± 1,228; significance was set at $P<0.016$). How-

ever, no significant differences were observed for primary (2,040 ± 849 from Arnt fl/fl; MC and 2,620 ± 817 from Arnt fl/fl) and preantral/antral (7,560 ± 4,582 from Arnt fl/fl; MC and 4,980 ± 1,582 from Arnt fl/fl) follicles. To investigate the possibility that the reduction in primary follicles was associated with an altered estrous cycle, we compared the estrus cycles in Arnt fl/fl; MC and Arnt fl/fl females. No differences were observed.

To determine whether the *Arnt* gene has been inactivated in ovarian tissue, we performed PCR analysis to identify the recombined Arnt allele. The recombined allele was detected (data not shown), which agrees with earlier studies that the MMTV-Cre transgene is active in the ovary (Riedlinger *et al.,* 2002; Wagner *et al.,* 1997). To determine whether the impaired fertility and ovarian dysfunction could be explained by altered hormone levels, we measured estradiol and progesterone levels in pseudopregnant mice. No differences were observed in estradiol levels from Arnt fl/fl; MC (23 ± 2 pg/ml) and Arnt fl/fl (25 ± 3 pg/ml) mice $P = 0.69$. Similarly, no differences were observed in progesterone levels from Arnt fl/fl; MC (31 ± 5 ng/ml) and Arnt fl/fl (36 ± 9 ng/ml) mice, $P = 0.63$.

AhR Is Not Required for the Development of the Mammary Gland

It has been reported that ductal development and lactation are impaired in AhR-null mice (Hushka *et al.,* 1998; Abbott, 1999). Since this could be the result of impaired ovarian function of AhR-null mice (Benedict *et*
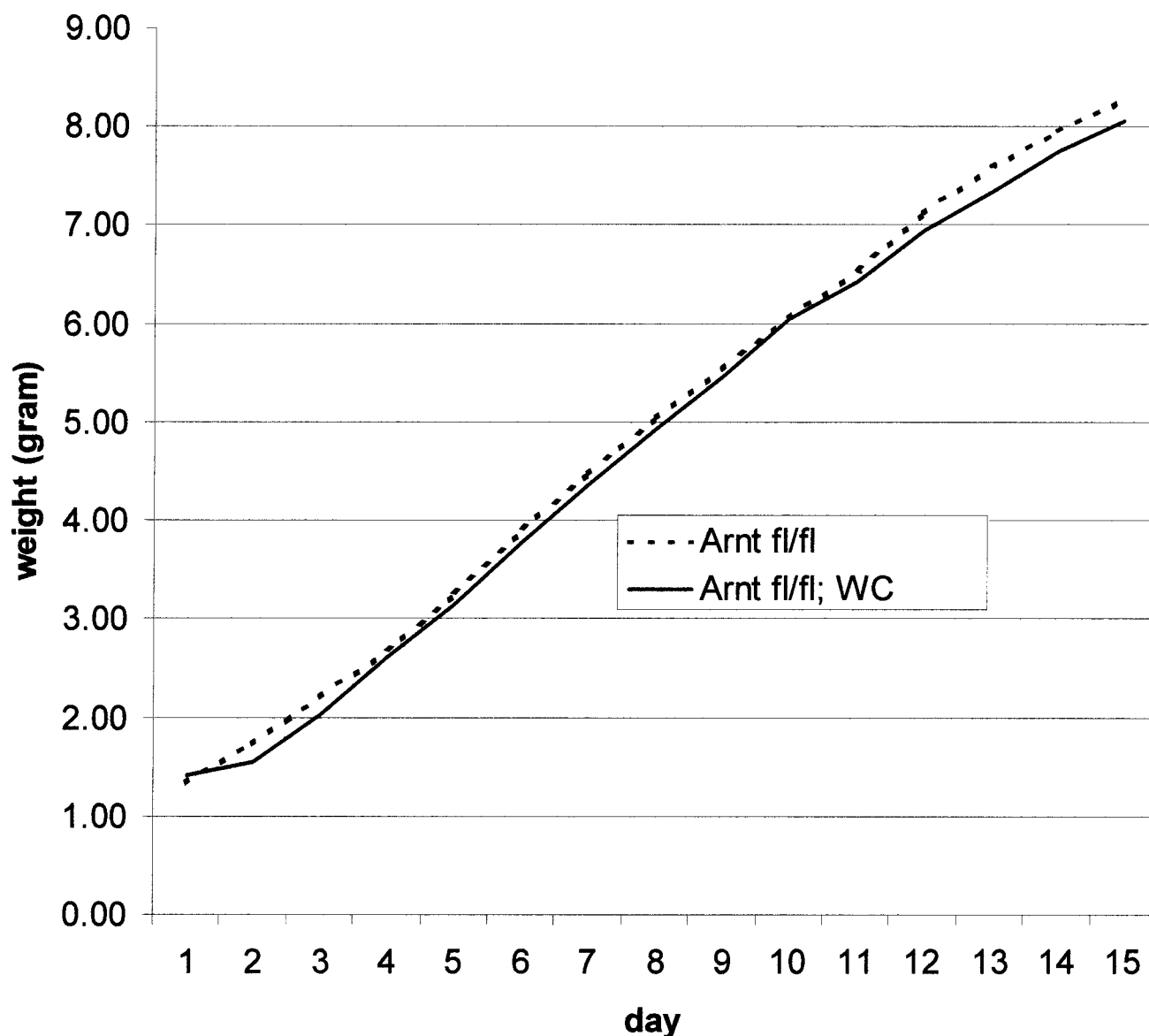
FIG. 3. Weight gain of pups fed by Arnt fl/fl; WC dams (solid line) and by Arnt fl/fl dams (dashed line) during 15 days after birth. Each weight data is the average of 48 pups' weight (6 litters with 8 pups each).

al., 2000), we investigated mammary development upon transplantation of AhR-null epithelium into cleared fat pads of wild-type mice. Mammary tissue was harvested during puberty and at parturition, followed by whole-mount and histological analyses (Fig. 6). No developmental differences were observed between mammary tissue derived from control (AhR +/+) and AhR-null epithelium.

DISCUSSION

The AhR and Arnt are members of the bHLH-PAS family of transcription factors that play a role in the cell's ability to respond to environmental cues. Antiestrogenic effects

of AhR-Arnt signaling have been reported, suggesting that this pathway could participate in the development of organs that are under control of steroid hormones. We have detected high levels of Arnt in thymus, lung, liver, ovary, virgin mammary tissue, and the fat pad underlying the mammary gland but not in spleen and kidney. This is different from RNA studies that reported ubiquitous presence of Arnt RNA (Hirose *et al.,* 1996). In mammary tissue, Arnt levels were similar throughout puberty, pregnancy, and lactation and low prior to puberty and during involution. This suggested a role for Arnt during key developmental stages. We inactivated the *Arnt* gene within the alveolar compartment during pregnancy with the WAP-Cre transgene. These mice were fertile, gave
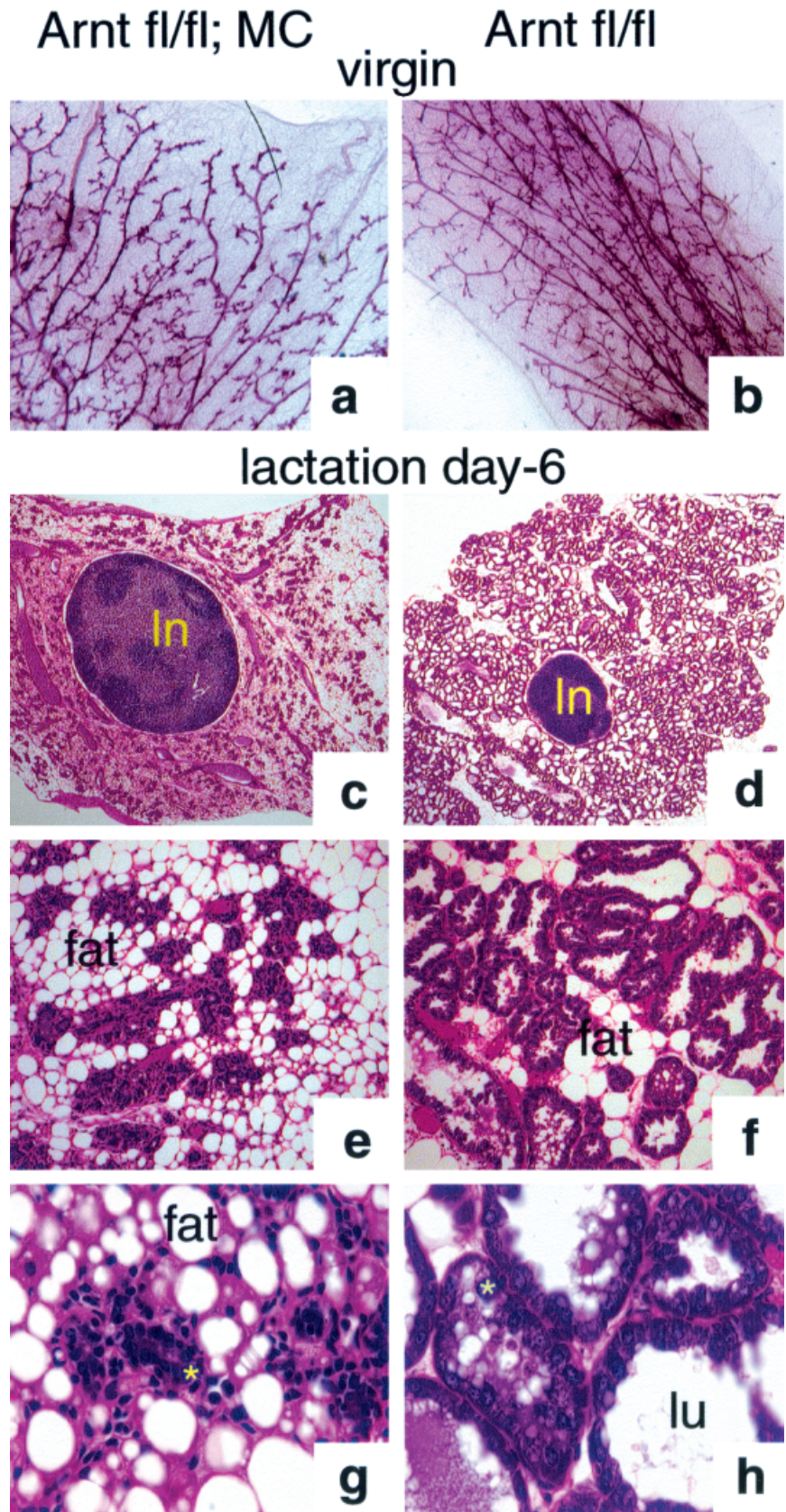
FIG. 4. Histological analyses of mammary tissue from Arnt fl/fl; MC and Arnt fl/fl mice. **(a,b)** Mammary whole mounts from 3-month-old Arnt fl/fl; MC and Arnt fl/fl virgins; **(c,e,g)** mammary tissues harvested at day 6 of lactation from Arnt fl/fl; MC; **(d,f,h)** mammary tissues harvested at day 6 of lactation from Arnt fl/fl mice. Magnification 12× (a,b); 25× (c,d); 200× (e,f); 630× (g,h). ln, lymph node; *, epithelial cell; lu, lumen.
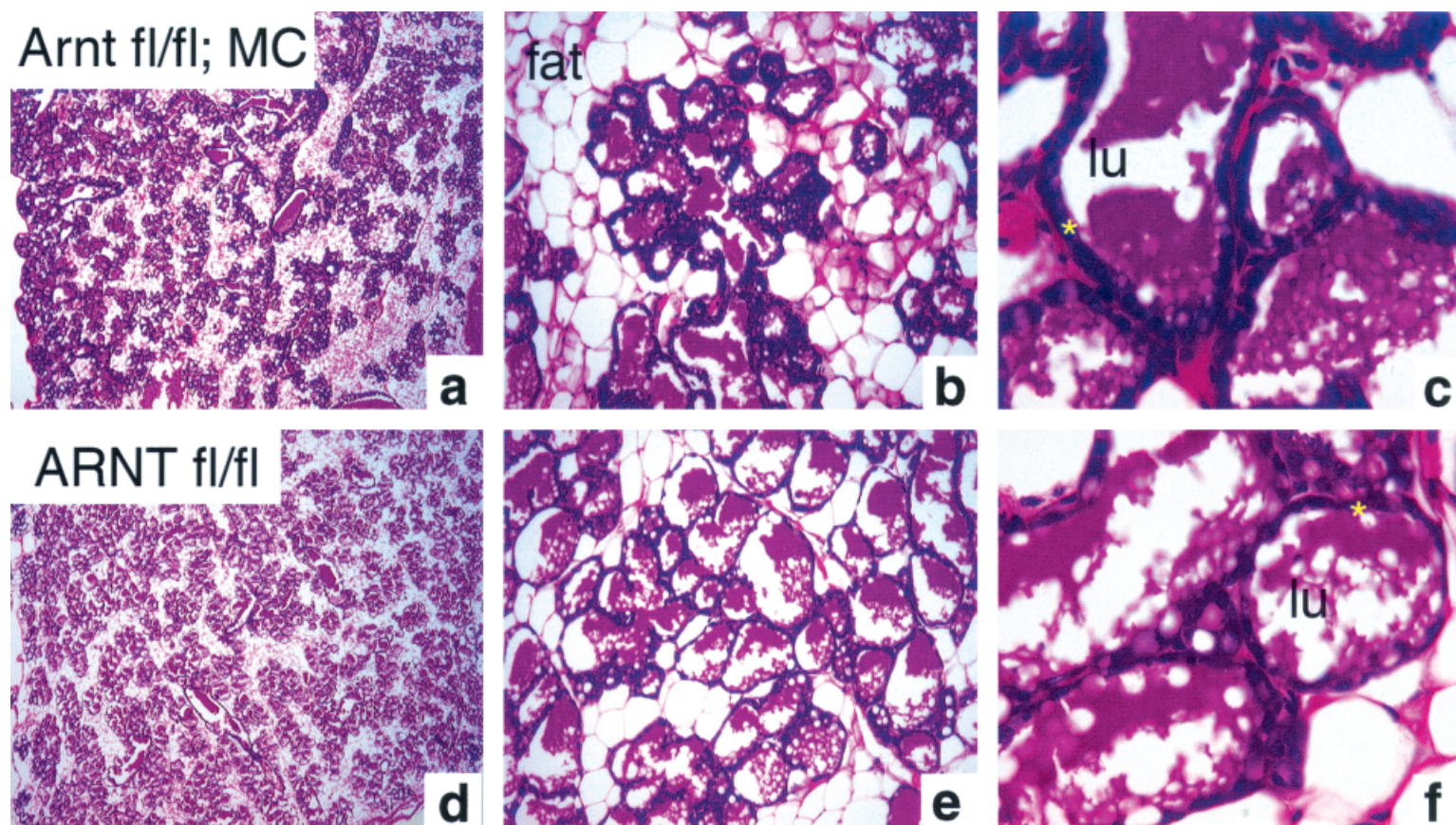
FIG. 5. Histology of Arnt fl/fl; MC **(a–c)** and Arnt fl/fl **(d–f)** mammary epithelium transplanted into wild-type mice at day 1 of lactation after one pregnancy. Magnification 25× (a,d); 200× (b,e); 630× (c,f). *, epithelial cell; lu, lumen.

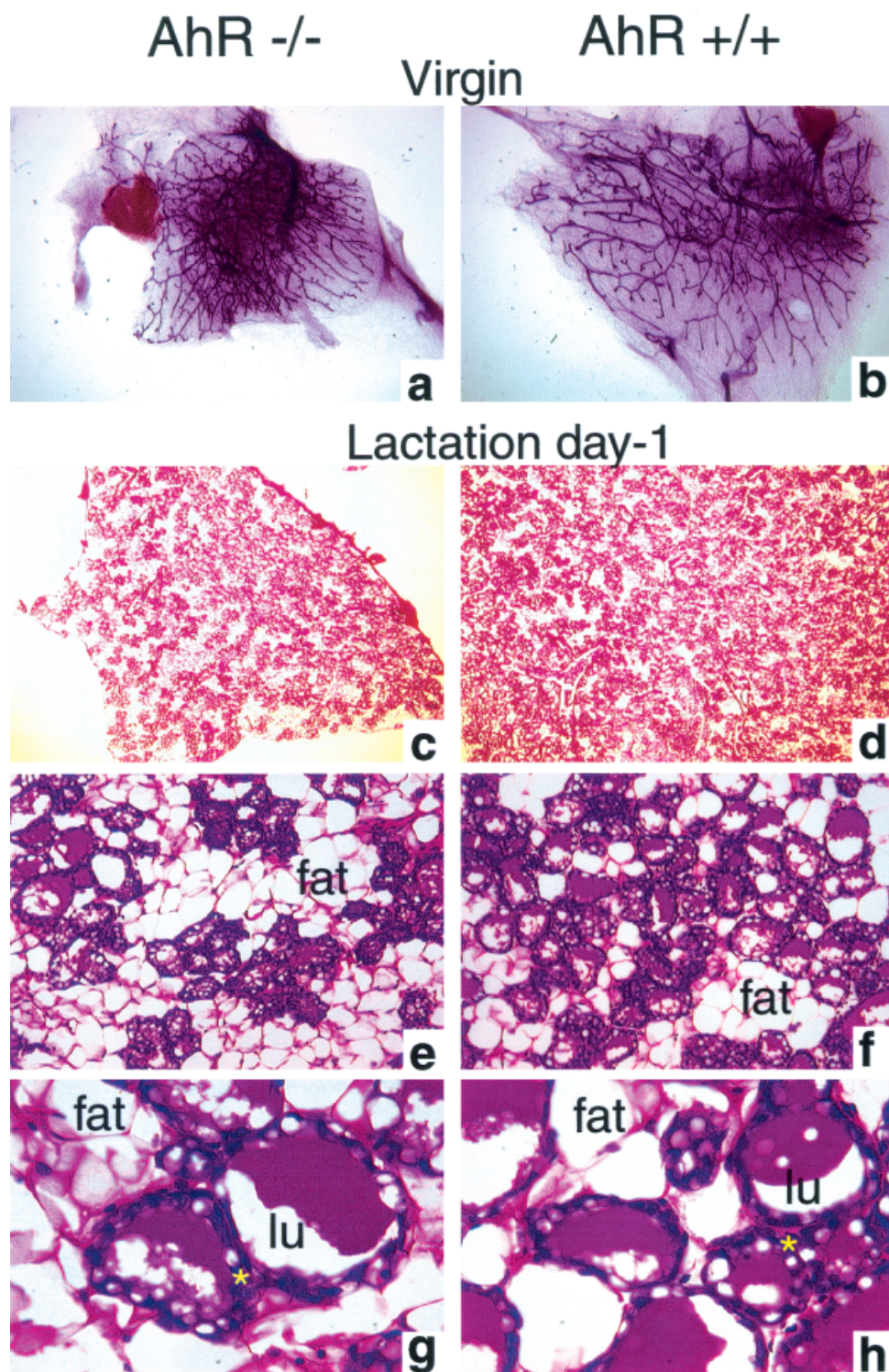## AhR -/-   AhR +/+
Virgin

Lactation day-1

FIG. 6. Histology of AhR −/− **(a,c,e,g)** and AhR +/+ **(b,d,f,h)** mammary epithelium transplanted into wild-type mice. (a,b) Mammary tissues harvested from virgin (7 weeks after transplant experiment); (c–h) mammary tissues harvested at day 1 of lactation after one pregnancy. Magnification 12× (a,b); 25× (c,d); 200× (e,f); 630× (g,h). *, epithelial cell; lu, lumen.

birth to normal-sized litters, and the dams were able to raise their young. No histological abnormalities or functional deficiencies were observed. However, when the *Arnt* gene was inactivated during the early stages of mammary gland development using the MMTV-Cre transgene, retarded mammary development, impaired mam-

mary function, and small litter sizes were observed. Arnt-null mammary epithelium transplanted into wild-type mice developed normally, suggesting that the developmental lesions were possibly due to impaired ovarian function. This was supported by a decrease in primordial follicles in mice in which the *Arnt* gene had been inactivated in the majority of granulosa and luteal cells. Progesterone and estrogen levels were normal in these mice and the reason for their impaired fertility remains unknown. Since Arnt is also a binding partner for hypoxia-inducible factor (HIF), which affects homeostasis and angiogenesis, this pathway could contribute to ovarian function, as seen in liver (Tomita *et al.,* 2000).

Arnt is expressed highly in mammary tissue, and the lack of developmental defects in its absence could be the result of compensation by other members of this family, most notably Arnt2, which can form functional complexes with the AhR (Hirose *et al.,* 1996). However, Arnt2 is only moderately conserved with Arnt and only expressed in a few tissues (Hirose *et al.,* 1996) and we have not detected expression in mammary tissue. Alternatively, it is possible that the function of Arnt in mammary epithelium can only be seen when the mouse is exposed to environmental toxicants. Since the mammary stroma also expresses Arnt, and both Cre stains target only the epithelial compartment of the gland, we cannot rule out a role for Arnt through the stroma.

AhR is the dimerization partner of Arnt and its presence is required for normal ovarian follicles growth (Benedict *et al.,* 2000) and development of mammary ducts during puberty (Hushka *et al.,* 1998). These investigators demonstrated a 50% reduction in terminal end buds and an increase in blunt-ended terminal ducts in AhR-null mice. However, AhR-null mammary epithelium forms normal ducts and alveoli when transplanted into wild-type mice. This demonstrates that the AhR is not required for development the epithelial compartment and that the effect seen by others is probably due to altered levels of systemic hormone.

MATERIALS AND METHODS

Transgenic Mice

Conditional Arnt-null mice had been generated by inserting *loxP* sites into introns 5 and 6 (Tomita *et al.,* 2000). These mice were mated with WAP- and MMTV-Cre transgenic mice (Wagner *et al.,* 1997). The genotype of the mice was determined by PCR analyses. Primers for *Arnt* gene were F2 (5′-tgccaacatgtgccaccatgt-3′) and R2 (5′-gtgaggcagatttcttccatgctc-3′), which yielded a 290-bp product from the wild-type allele and a 340-bp product from the floxed allele after 30 cycles of 45 sec at 95°C, 45 sec at 60°C and 45 sec at 72°C and 1 cycle of 5 min at 72°C.

Primers for the Cre transgenes were 5′-tagagctgtgc-cagcctcttcc-3′, which binds in the WAP gene promoter; 5′-ggttctgatctgagctctgagtg-3′, which binds in the MMTV-LTR; and 5′-catcactcgttgcatcgaccgg-3′, which binds in the Cre sequence. Using the respective promoter prim-

ers with the Cre primer, the WAP-Cre transgene produced a 240-bp fragment after 30 cycles of 45 sec at 95°C, 45 sec at 58°C and 1 min at 72°C and 1 cycle of 5 min at 72°C. All products were separated in 2.5% agarose TAE gels.

PCR assays were performed to determine the Arnt genomic deletion in ovaries, using primers R2, R6 (5′-ggctatacagtgcagcatggact-3′) and F4 (5′-acgcactacaacacct-gagctaa-3′) with the same cycling conditions as described for genotyping. A 240-bp product from the wild type, a 359-bp product from floxed allele, and a 317-bp product from the deleted fragment were used.

AhR-null mice were originally generated as described by Schmidt *et al.* (1996). The genotype of the AhR-null mice was determined as described previously (Benedict *et al.,* 2000).

For all mice studied, the fourth mammary glands were biopsied and the lymph nodes were surgically excised at the time of the mammary gland biopsies.

Western Blot Analysis

Tissues were homogenized in ice-cold lysis buffer consisting of 40 mM Tris.HCl (pH 8.0), 280 mM NaCl, 20% glycerol, 1% NP-40, 4 mM EDTA, 20 mM NaF, 100 μg/ml PMSF, 40 μg/ml aprotinin, 40 μg/ml leupeptin, and 2 mM Na-vanadate. Protein concentration was determined using the Bio-rad protein assay kit with BSA as a standard. Twenty or 40 μg of protein were loaded per lane, separated by NuPAGE Tris Acetate on a 7% gel (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen) The membranes were blocked overnight at 4°C with TBST (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween-20) containing 3% nonfat dry milk and incubated for 1 h with mouse anti-Arnt (611078, BD Transduction Laboratories), rabbit anti-Stat5b serum (Liu *et al.,* 1996), mouse anti-actin (Chemicon International, Temecula, CA), diluted at 1:1,000, 1:10,000, or 1:5,000, respectively, with 3% nonfat dry milk in TBST. HRP-conjugated goat anti-rabbit or rabbit anti-mouse IgG were used as secondary antibody and reacted at 1:5,000 with 3% nonfat dry milk in TBST for 45 min. Bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Histological Evaluation of Ovaries and Mammary Glands

Ovaries were fixed in Bouin's solution overnight and then washed in 70% ethanol. Paraplast (VWR Scientific, Buffalo Grove, IL) embedded ovaries were serial sectioned (8 μm) through the entire tissue, mounted on glass slides, and stained with Weigert's hematoxylin-picric acid methylene blue. Every tenth section was analyzed for primordial, primary, and preantral/antral follicle numbers. The number of follicles in every tenth section was multiplied by 8 to give an estimate of the total follicle numbers. Only the follicles with a visible nucleus in the oocyte were counted to avoid double counting.

The mammary gland (no. 4) was removed at the indicated times of development and spread on a glass slide. After fixation for 4 h in Carnoy's solution, the glands were hydrated and stained with carminalum, dehydrated, and mounted as described by Kordon *et al.* (1995). The glands were photographed, paraffin embedded, and sectioned at 5 μm. Sections were stained with hematoxylin and eosin.

Mammary epithelium transplantation

The endogenous epithelium of athymic nude 3-week-old mice was removed as described by DeOme *et al.* (1959). An incision was made in the cleared fat pad and a small piece of the donor mammary gland was transplanted. For study of Arnt function, mammary tissue from Arnt fl/fl; MC and Arnt fl/fl mice were transplanted in the right and the left sides, respectively. For study of AhR function, mammary tissue from AhR −/− and AhR +/+ mice were transplanted in the right and the left sides, respectively. Eight weeks after surgery, the host mice were mated and the transplanted tissues were harvested the delivery day. A detailed description of the transplantation of adult tissue into fat pads can be found at http://mammary.nih.gov/tools/mousework/index.html.

Hormone Levels

Estradiol and progesterone levels were measured by RIA using Coat-A-Count (Diagnostic Products Corporation, Los Angeles, CA). Mice were anaesthetized and blood was collected by phlebotomy from the retro-orbital plexus. Serum was separated from cells by centrifugation for 5 min at 5,000 rpm. Serum from four 8-week-old Arnt fl/fl; MC and four Arnt fl/fl pseudopregnant mice (intraperitoneal injection of 5 IU of PMSG followed 48 h later by intraperitoneal injection of 5 IU of hCG every 24 h for 72 h) was analyzed.

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